

Silencing of *FABP3* Inhibits Proliferation and Promotes Apoptosis in Embryonic Carcinoma Cells

Yahui Shen · Guixian Song · Yaoqiu Liu ·
Lijuan Zhou · Hailang Liu · Xiangqing Kong ·
Yanhui Sheng · Kejiang Cao · Lingmei Qian

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Abstract Fatty acid-binding protein 3 (*FABP3*) facilitates the movement of fatty acids in cardiac muscle. Previously, we reported that *FABP3* is highly upregulated in the myocardium of ventricular septal defect patients and overexpression of *FABP3* inhibited proliferation and promoted apoptosis in embryonic carcinoma cells (P19 cells). In this study, we aimed to investigate the effect of *FABP3* gene silencing on P19 cell differentiation, proliferation and apoptosis. We used RNA interference and a lentiviral-based vector system to create a stable *FABP3*-silenced P19 cell line; knockdown of *FABP3* was confirmed by quantitative real-time PCR. Expression analysis of specific differentiation marker genes using quantitative real-time PCR and observation of morphological changes using an inverted microscope revealed that knockdown of *FABP3* did not significantly affect the differentiation of P19 cells into cardiomyocytes. CCK-8 proliferation assays and cell cycle analysis demonstrated that *FABP3* gene silencing significantly inhibited P19 cell proliferation. Furthermore, Annexin V-FITC/propidium iodide staining and the caspase-3 activity assay revealed that *FABP3* gene silencing significantly promoted serum starvation-induced apoptosis in P19 cells. In agreement with our previous research, these

results demonstrate that *FABP3* may play an important role during embryonic heart development, and that either overexpression or silencing of *FABP3* will lead to an imbalance between proliferation and apoptosis, which may result in embryonic cardiac malformations.

Keywords *FABP3* · Congenital heart disease · RNA interference · P19 cells

Introduction

Heart development is controlled by an evolutionarily conserved network of transcription factors which connect the signaling pathways and genes responsible for muscle growth, patterning and contractility, and involves a precisely orchestrated series of molecular and morphogenetic events. Mutations in components of the cardiac gene network or subtle perturbation of the processes which these genes regulate can have catastrophic consequences in the form of congenital heart disease (CHD) [1]. CHD is currently the most common type of birth defect, with an incidence ranging from approximately 4 to 50 per 1,000 live births [2, 3]. Although the mortality rate of CHD has decreased due to advances in medical and surgical management, not all CHD patients survive to adulthood.

Ventricular septal defect (VSD) is the most common form of CHD and among the most frequently observed congenital abnormalities [4]. The ontogeny of ventricular septation is complex and requires coordinated proliferation, specification, differentiation, migration, apoptosis, morphogenesis and cellular interactions [5]. As a multifactorial complex disease, a number of environmental and genetic factors have been identified to play an important role in the occurrence of VSD. With the recent development of new

Yahui Shen, Guixian Song contributed equally to this work.

Y. Shen · Y. Liu
State Key Laboratory of Reproductive Medicine, Department of MICU, Nanjing Maternity and Child Health Care Hospital Affiliated to Nanjing Medical University, Nanjing 210029, China

G. Song · L. Zhou · H. Liu · X. Kong · Y. Sheng · K. Cao · L. Qian (✉)
Department of Cardiology, The First Affiliated Hospital of Nanjing Medical University, Nanjing 210029, China
e-mail: lmqian@njmu.edu.cn

techniques in molecular and developmental biology, the past decade has witnessed spectacular progress in elucidation of the molecular mechanisms involved in CHD. However, so far, very few of the genes which cause VSD have been identified. Therefore, the identification and characterization of novel genes and proteins associated with VSD remains an issue of intense interest.

Fatty acid-binding protein 3 (FABP3) is a low molecular weight protein with a distinct tissue-specific distribution pattern, which plays an important role in fatty acid transport, cell growth, cellular signaling and gene transcription [6, 7]. In previous studies using suppression subtractive hybridization [8], we found that *FABP3* is highly expressed in the myocardium of VSD patients. Further studies revealed that overexpression of *FABP3* inhibited proliferation and promoted apoptosis in embryonic myocardial cells and affected the differentiation of cardiac precursors into mature cardiomyocytes [9]. Therefore, in the present study, we aimed to investigate whether *FABP3* gene silencing is capable of enhancing proliferation, inhibiting apoptosis and affecting the differentiation of embryonic myocardial cells. The P19 cell line is a suitable model for cardiac differentiation at the molecular and functional levels [10]; therefore, we selected P19 cells to investigate the impact of *FABP3* gene silencing on embryonic myocardial cell differentiation, proliferation and apoptosis.

Materials and Methods

Cell line and Cell Culture

P19 cells (embryonic carcinoma cells) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells (the sixth generation) were cultured in α -modified Eagle's medium (α -MEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin and 100 μ g/ml streptomycin at 37 °C in 5 % CO₂. In order to induce cardiac differentiation, P19 cells were cultivated as aggregates from days 0 to 4 in the presence of α -MEM

containing 10 % FBS and 1 % DMSO in bacteriological dishes at 37 °C in 5 % CO₂, as previously described [11]. At day 4, the embryoid bodies were transferred to 6-cm culture flasks and then cultured in α -MEM containing 10 % FBS for an additional 4 or 6 days. The morphological changes in P19 cells were examined and photographed using an inverted microscope (Nikon, Japan).

Construction of RNAi Lentiviral Vector

A short hairpin RNA (shRNA) construct was designed to contain a unique 19-nt double-stranded *FABP3* sequence presented as an inverted complementary repeat, a loop sequence (5'-CTCGAG-3'), the RNA PolIII terminator (5'-TTTTTT-3') and 5' single-stranded overhangs for ligation into AgeI- and EcoRI-digested pGLV-U6-Puro lentivirus vector (GenePharma, Shanghai, China). The recombinant vector was named pGLV-*FABP3*-shRNA. The negative control vector (pGLV-NC-shRNA) contained a nonsense shRNA insert in order to control for effects due to non-RNAi mechanisms. The sequences of the two cDNA fragments (positive-sense strand) were as follows: *FABP3*, 5'-CATCCTGACTCTCACTCAT-3'; negative control, 5'-TTCTCCGAACGTGTACAGT-3'.

Virus Generation and Infection

The lentiviral supernatant was produced by transient cotransfection of HEK-293 T cells with the pGLV-*FABP3*-shRNA or pGLV-NC-shRNA lentiviral plasmids with three optimized packaging plasmids: a packing plasmid (pGag/Pol), a VSV-G-expressing plasmid (pVSV-G) and a Rev (pRev)-expressing plasmid, using the method of Chen and Okayama [12]. The supernatants were collected 48 h after transfection, filtered through a 0.45 μ m membrane, supplemented with hexadimethrine bromide (polybrene) to a final concentration of 5 μ g/ml and applied directly to P19 cells. Stably transduced cells were selected using puromycin, by adding the minimum concentration of puromycin required to kill untransduced cells. The efficiency of knockdown was analyzed by quantitative real-time polymerase chain reaction (qPCR) using the primers shown in Table 1.

Table 1 Oligonucleotide sequences for the primer sets used in qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
<i>FABP3</i>	ATGAGAAGGAGGCGTGAC	ATCCCAACCCAAGAATGT
<i>GAPDH</i>	ATTCAACGGCAGTCAA	CTCGCTCCTGGAAGATGG
<i>cTnT</i>	GAAGGAAAGGCAGAACCG	ACCCTCCAAAGTGCATCA
<i>GATA4</i>	ACGGAAGCCCAAGAACCTG	GCTGCTGTGCCCATAGTGAG
<i>NKX2.5</i>	GACAGCGGCAGGACCAGACT	GCCATAGGCATTGAGACCCAC
α -MHC	CACCGTGGACTACAACATTAT	GCTTGAGGGAGGACTTCTG

Quantitative Real-Time Reverse Transcription PCR

Quantitative real-time reverse transcription PCR (qRT-PCR) was performed to determine the expression of marker genes during P19 cell differentiation. Total RNA was extracted from P19 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized from 1 µg total RNA using the AMV Reverse Transcriptase Kit (Promega, Madison, WI, USA). Real-time PCR was performed using the SYBR green method in an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA, USA), following the manufacturer's protocols. The sequences of the primers used are shown in Table 1. The PCR conditions included a denaturation step (95 °C for 10 min), followed by 40 cycles of amplification and quantification (95 °C for 15 s, 60 °C for 1 min). Relative gene expression levels were quantified based on the cycle threshold (C_t) values and normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each sample was measured in triplicate, and the gene expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method, as described previously [13].

Cell Growth Assay

Cell growth was analyzed using the WST-8 Cell Counting Kit-8 (Dojindo, Tokyo, Japan) according to the manufacturer's instructions. Briefly, the cells were seeded in 96-well plates and incubated in α -MEM supplemented with 10 % FBS for 7 days. On the day of growth measurement, 100 µl of the spent media was replaced with an equal volume of fresh media containing 10 % CCK-8, and the cultures were incubated at 37 °C for 120 min. Absorbance was measured at 450 nm using a microplate reader. The results were plotted as the mean \pm SD of three separate experiments with three determinations per experiment for each experimental condition.

Cell Cycle Analysis

Cell cycle distribution was analyzed using flow cytometry. Before analysis, the cells were serum deprived for 24 h to synchronize the cell cycle, then the cells were incubated with α -MEM containing 10 % FBS for 24 h after serum deprivation. The cells were trypsinized, rinsed with phosphate-buffered saline (PBS), fixed with 70 % ethanol at 4 °C overnight and treated with RNaseA (0.02 mg/ml) in the dark at room temperature for 30 min. The cells were resuspended in 0.05 mg/ml propidium iodide (PI; Sigma, St. Louis, MO, USA) and analyzed by flow cytometry (BD Biosciences, USA). At least 1×10^4 cells were recorded for each sample, and the data were analyzed using Cell Quest software (BD Biosciences).

Quantification of Apoptosis

Cells were cultured in serum-free α -MEM for 24 h to induce apoptosis, and then cultured in complete medium containing 10 % FBS for 0 or 24 h, harvested using trypsin, washed with PBS, centrifuged, resuspended in 1 ml binding buffer and stained with 10 µl Annexin V-FITC and 10 µl PI at room for 15 min (Biovision, California, CA, USA). The stained cells were analyzed immediately using flow cytometry as previously described [14]. Cell Quest software was used for data acquisition and analysis, and the percentage of apoptotic cells was determined.

Measurement of Caspase-3 Activity

Cells were cultured in serum-free α -MEM for 24 h to induce apoptosis, then collected and washed with PBS. Caspase-3 activity was assayed using a caspase-3 colorimetric assay kit (KeyGen, Nanjing, China) according to the manufacturer's protocol. Briefly, 5×10^6 cells were lysed in 50 µl chilled cell lysis buffer on ice for 20 min, centrifuged for 1 min at 10,000 rpm at 4 °C. Aliquots of the supernatant containing 200 µg protein were diluted to 50 µl with cell lysis buffer, incubated with 5 µl substrate at 37 °C for 4 h, and the absorbance value of the samples were read at 405 nm in a microtiter plate reader.

Statistical Analysis

Each experiment was performed at least three times. The data are presented as the mean \pm standard deviation (SD) values. All data were analyzed by the Student's t test using the SPSS 13.0 statistical package (SPSS, Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

Results

Creation of A Stable *FABP3*-silenced P19 Cell Line

The knockdown of *FABP3* was evaluated by real-time PCR. The relative expression of *FABP3* mRNA in P19 cells was normalized to the endogenous control gene GAPDH. As shown in Fig. 1, P19 cells transfected with lentiviral *FABP3*-shRNA expressed significantly lower levels of *FABP3* mRNA than the cells transfected with the negative control (NC) lentiviral construct ($*P < 0.05$). The steady state *FABP3* mRNA expression level in the lentiviral-*FABP3*-shRNA-transfected cells was 76 % of NC cells.

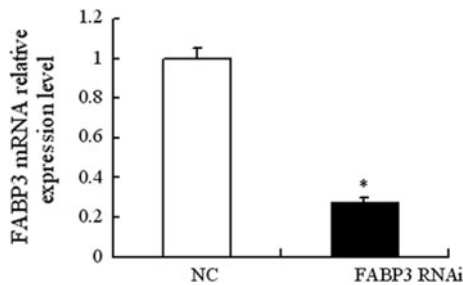


Fig. 1 Quantitative RT-PCR confirmation of *FABP3* gene silencing in pGLV-*FABP3*-shRNA-transduced P19 cells. *FABP3* mRNA expression was inhibited by 76 % in P19 cells transfected with pGLV-*FABP3*-shRNA (*FABP3* RNAi), compared to cells transfected with control pGLV-NC-shRNA lentivirus (NC). Values are the mean \pm SD of three independent experiments (* $P < 0.05$)

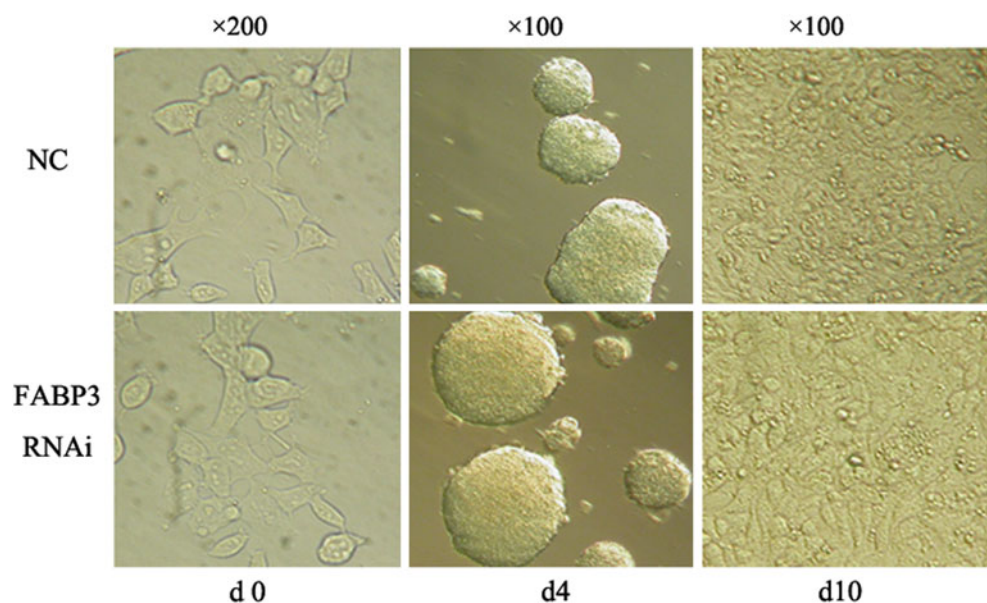
FABP3 Gene Silencing has No Effect on the Morphology of P19 Cells During Differentiation

To investigate the effect of *FABP3* gene silencing on the differentiation of P19 cells into cardiac cells, we examined the morphological changes in P19 cells during DMSO-induced differentiation. The morphology of P19 cell on days 0, 4 and 10 of differentiation are shown in Fig. 2. The cardiac myocardial cells aggregated on day 4 of differentiation, and beating cell colonies appeared on day 10 of differentiation. However, no differences were observed in the morphological changes or time taken for the appearance of beating cell clusters in NC cells and lentiviral-*FABP3*-shRNA-transfected cells.

FABP3 Gene Silencing has No Effect on the Expression of Marker Genes During P19 Cell Cardio-differentiation

ES (embryonic stem) cell-derived cardiomyocytes express cardiac-specific genes and proteins in a developmentally

Fig. 2 *FABP3* gene silencing has no effect on the morphology of P19 cells during P19 cell cardio-differentiation. P19 cells were transfected with pGLV-*FABP3*-shRNA (*FABP3* RNAi) or pGLV-NC-shRNA lentivirus (NC) and treated with 1 % DMSO to induce cardiomyocyte differentiation over 10 days. No obvious morphological differences were observed in *FABP3*-silenced and NC cells at days 0, 4 and 10



controlled manner. For example, induction of cardiac troponin T (*cTnT*) mimics the isoform transition which occurs during cardiogenesis in vivo. The early cardiac transcription factor *Nkx2.5*, *GATA4* and the cardiac muscle-specific marker α -*MHC* [15] were used as cardiac-specific markers in this study. We used qRT-PCR to analyze the expression levels of these genes during differentiation. As shown in Fig. 3, the expression of all the marker genes gradually increased during the process of differentiation; however, no significant difference in the mRNA expression levels of any marker gene was observed in *FABP3*-silenced cells and NC cells at any time point (days 0, 6, 8 and 10; $P > 0.05$).

FABP3 Gene Silencing Reduces P19 Cell Proliferation

The growth of *FABP3*-silenced and NC P19 cells was assessed using the CCK-8 cell viability assay. As shown in Fig. 4a, *FABP3*-silenced cells had a reduced growth rate compared to NC control cells. At days 1, 2 and 3, the OD values of *FABP3*-silenced and NC control cells were not significantly different. However, from day 4 onwards, the OD values of *FABP3*-silenced cells were significantly lower than control cells (* $P < 0.05$).

We also performed flow cytometry analysis of the cell cycle distribution in *FABP3*-silenced and NC control P19 cells, and observed that the proportion of S phase cells in *FABP3*-silenced P19 cells was much lower than NC control cells (Fig. 4b).

FABP3 Gene Silencing Induces P19 Cell Apoptosis

Annexin V-FITC binds to phosphatidylserine which can be used to detect the early stages of apoptosis [14]. Apoptosis was induced by serum starvation for 24 h; then, the cells

Fig. 3 *FABP3* gene silencing has no effect on the expression of marker genes during P19 cardiomyocyte differentiation. P19 cells were transfected with pGLV-*FABP3*-shRNA (*FABP3* RNAi) or pGLV-NC-shRNA lentivirus (NC) and treated with 1 % DMSO to induce cardiomyocyte differentiation, as described in the Materials and Methods. The expression of specific differentiation markers (*cTnT*, *GATA4*, *Nkx2.5* and α -MHC) was determined by quantitative real-time PCR at various time points during differentiation (days 0, 6, 8 and 10). Values are the mean \pm SD of three independent experiments ($n = 6$; $P > 0.05$)

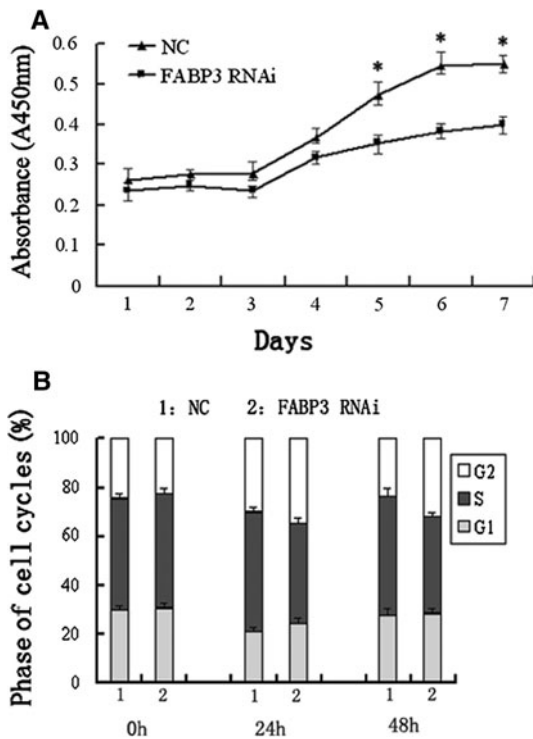
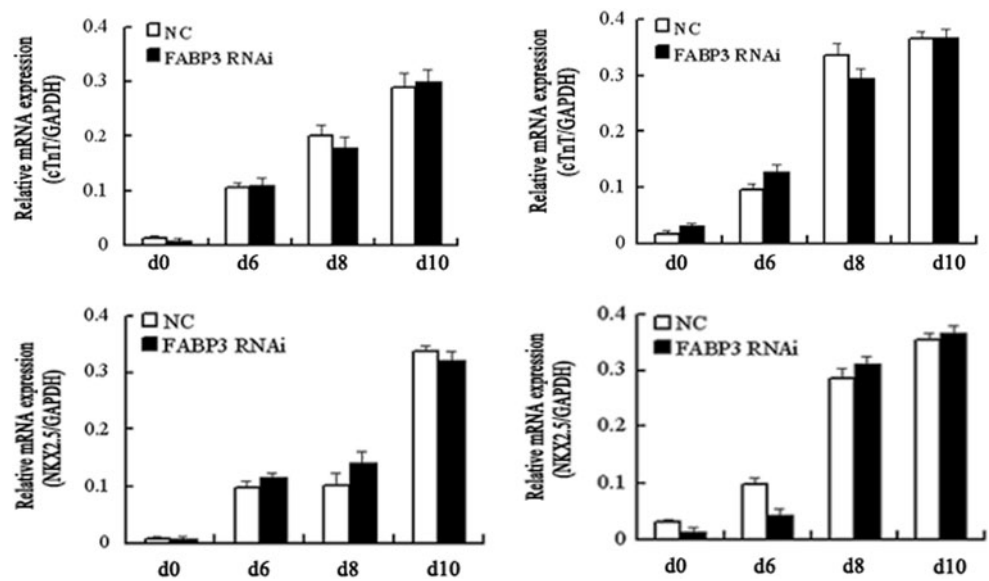


Fig. 4 *FABP3* gene silencing reduces P19 cell proliferation. P19 cells were transfected with pGLV-*FABP3*-shRNA (*FABP3* RNAi) or pGLV-NC-shRNA lentivirus (NC). Cell growth was quantified using the CCK-8 cell viability assay (a) and cell cycle analysis (b). Values are the mean \pm SD of three independent experiments ($*P < 0.05$)

were cultured in complete media for 0 or 24 h, and the numbers of apoptotic cells were quantified by flow cytometric analysis after staining with Annexin V-FITC/PI. *FABP3* gene silencing increased the number of apoptotic cells in response to serum deprivation ($*P < 0.05$; Fig. 5a, b). Additionally, the caspase-3 activity assay (Fig. 5c) also

indicated that *FABP3* gene silencing increased the number of apoptotic cells in response to serum deprivation. These results indicate that *FABP3* gene silencing promotes serum deprivation-induced apoptosis.

Discussion

Observational epidemiological studies have demonstrated the existence of genetic predispositions for CHD. Although many genes have been found to be important for cardiac development in animal studies, to date, only a few human genes have been associated with CHD as critical regulators of heart formation, including *NKX2.5* [16], *Tbx5* [17] and *GATA4* [18]. Moreover, for most types of CHD, the basic pathogenetic mechanisms of VSD remain poorly characterized.

Previous studies have shown that *FABP3* is expressed and upregulated in mouse neonatal hearts during cardiomyocyte terminal differentiation [19]. In present study, we used the P19 cell line as a research model, by establishing a stable line of *FABP3*-silenced P19 cells. RNA interference (RNAi) is a form of posttranscriptional gene silencing mediated by short double-stranded RNAs, known as small interfering RNAs (siRNA). RNAi is a powerful gene silencing mechanism which operates in most eukaryotic cells [20]. Long-lasting RNAi-based gene silencing can be achieved with the help of lentivirus-based expression systems, which drive the production of short hairpin RNA species (shRNAs) under the control of the RPPH1 (encoding ribonuclease PRNA component H1) promoter [21–23]. Moreover, lentiviral vectors encoding antisense targeting sequences have been used in clinical trials with no obvious side effects [22]. In this study, we successfully

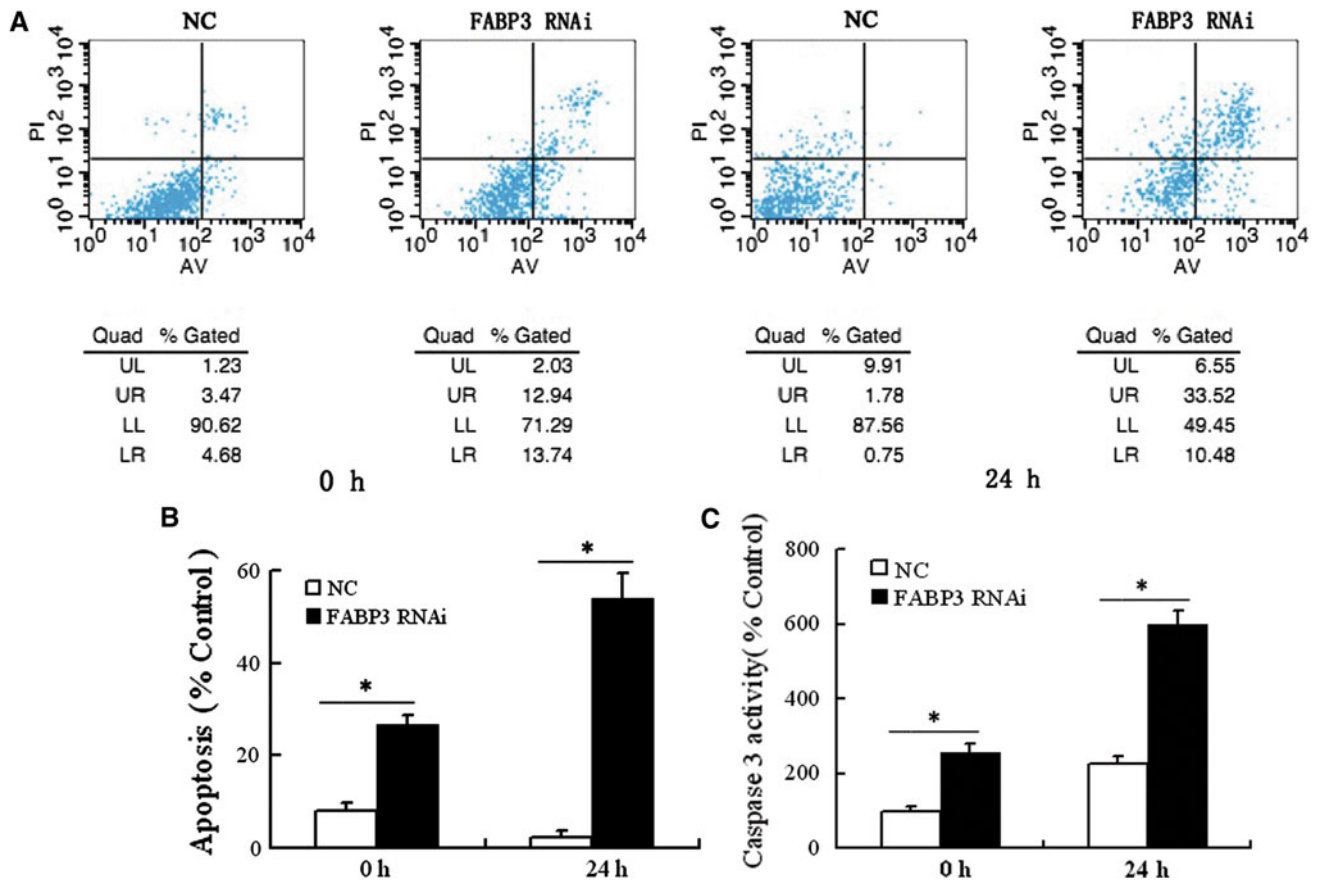


Fig. 5 *FABP3* gene silencing induces P19 cell apoptosis in response to serum starvation. P19 cells were transfected with pGLV-*FABP3*-shRNA (*FABP3* RNAi) or pGLV-NC-shRNA lentivirus (NC), serum starved for 24 h, then cultured in complete media for 0 or 24 h.

Apoptosis was quantified by flow cytometry using Annexin V-FITC/PI staining (a, b) and the caspase-3 activity assay (c). Values are the mean \pm standard deviation of three independent experiments ($n = 6$, $*P < 0.05$)

constructed an RNAi lentiviral vector to suppress endogenous expression of *FABP3*. The siRNA expression vector carrying antibiotics mark we employed can continuously inhibit the expression of target genes for several weeks in cells. Using real-time PCR technology, we confirmed that *FABP3* gene silencing resulted in a significant reduction in *FABP3* transcription. These effects were not observed in cells infected with the negative control shRNA.

We observed that knockdown of *FABP3* did not affect the differentiation of P19 cells into cardiomyocytes, as indicated by the morphological changes and expression of cardiomyogenesis-specific molecular markers. However, *FABP3* gene silencing dramatically inhibited proliferation and promoted apoptosis in P19 cells. In an earlier study, we also found that overexpression of *FABP3* inhibited proliferation, promoted apoptosis and affected the differentiation of cardiac precursors into mature cardiomyocytes [24], which suggests that overexpression or silencing of *FABP3* may influence morphogenesis in the embryonic heart by inducing excessive apoptosis in the myocardium.

In normal physical state, *FABP3* is expressed quantitatively in cardiac muscle tissue, involving in energy metabolism as translocators of long-chain fatty acid to intracellular organelles. So, whether ectopic expression or *FABP3*-RNAi will lead to the destruction of the physiological balance, and further influence myocardial energy metabolism which can induce mitochondrial impairment that further promote apoptosis [25, 26].

The heart is the first organ to form in the embryo, and a complex series of morphogenetic interactions involving cells from several embryonic origins is involved in the development of this organ [27]. Embryonic fetal heart growth depends on the balance between myocyte proliferation and apoptosis [28]. Inadequate proliferation or excess apoptosis can directly or indirectly result in CHD [29], which is often caused by altered proliferation and/or apoptosis in the septum, neighboring tissue or myocardium [30]. Our results demonstrate that knockdown of *FABP3* inhibited proliferation and promoted apoptosis in embryonic myocardial cells. We speculate that the molecular

mechanisms underlying this effect may be related to mitochondrial impairment as a result of *FABP3* gene silencing. *FABP3* participates in mitochondrial energy metabolism by transporting the fatty acids produced during the process of myocardial cell ATP generation. In addition, the mitochondrion has been regarded as a key element in apoptosis, as it plays a core role in the regulation of mammalian apoptosis [31]. We hypothesize that *FABP3* gene silencing downregulated *FABP3* protein expression, which affected energy metabolism in the cells and subsequently lead to mitochondrial dysfunction which, in turn, triggered apoptosis.

P19 cells, derived from an early mouse embryonic tumor, are pluripotent in nature and can differentiate into various cell types depending on the environment [32]. Cellular aggregation is known to be a requirement for the DMSO-induced differentiation of P19 cells into cardiac myocytes. Efficient differentiation of P19 cells depends on the prior formation of non-adherent aggregates which resemble the inner cell mass of an embryo; however, the molecular events that occur during aggregation and which are necessary for cardiac differentiation are not fully understood [10]. In this study, both *FABP3*-silenced and control P19 cells could be induced to differentiate into cardiac cells, as indicated by observation of the typical morphological changes during differentiation. Simultaneously, we also investigated the expression of cardiac muscle-specific molecular markers (*cTnT*, *GATA4*, *Nkx2.5* and α -*MHC*) during differentiation using quantitative real-time PCR. However, there was no significant difference in the mRNA expression levels of these marker genes in *FABP3*-silenced cells and NC cells at days 0, 6, 8 and 10. Thus, we suggest that *FABP3* may play a role in the regulation of cardiac cell proliferation and apoptosis; however, *FABP3* is not required for the differentiation of cardiac precursors into mature cardiomyocytes.

In summary, our results demonstrate that *FABP3* silencing promotes apoptosis and inhibits the proliferation of P19 cells. On the basis of previous research [9], our results suggest that both overexpression and silencing of *FABP3* can lead to an imbalance between proliferation and apoptosis, which may result in embryonic cardiac malformations. This study indicates that *FABP3* may be a potential target gene which could be modulated to prevent VSD. However, the detailed molecular mechanisms and signaling pathways which mediate the imbalance between proliferation and apoptosis in response to *FABP3* gene silencing or overexpression require further investigation in future studies.

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